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A Novel Method to Silence Genes in *Cryptosporidium*

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Abstract

The parasites of the genus *Cryptosporidium* are important causes of diarrheal diseases, specifically cryptosporidiosis, worldwide. A major bottleneck for developing drugs and vaccines against cryptosporidiosis is the lack of methods to study gene function in this parasite. Silencing of genes by RNA interference (RNAi) is a powerful method to investigate gene function that has been widely used in the identification of targets for several pathogens. Unfortunately, as *Cryptosporidium* does not possess the enzymes of the RNAi pathway, its genes cannot be silenced by standard siRNA technology. To circumvent that problem, we have developed a novel strategy to knock down *Cryptosporidium* genes by reconstituting the effector arm of the siRNA pathway. We have induced silencing of several genes in *Cryptosporidium* by transfecting parasites with hybrid complexes formed between recombinant human Argonaute (hAgo2) and *Cryptosporidium* single-stranded RNA (ssRNA). This novel methodology provides an effective strategy to study the role of selected genes in host–parasite interactions, and also can be used to identify potential targets for chemotherapy. The standardized methodology based on this strategy is described in this chapter.

Keywords

Cryptosporidium; Gene silencing; RNAi

1 Introduction

Gene silencing by RNA interference (RNAi) was described for the first time in 1998 [1]. RNAi technology is based on the use of small double-stranded RNA (siRNA) complementary to the mRNA target; once the siRNA is introduced into the cell it is processed by the RNA-induced silencing complex (RISC). During this process, the sense strand of the siRNA is removed and the anti-sense strand is used as guide to bind to the mRNA target. This association suppresses gene expression by inducing degradation of the targets [2], and this process of degradation is conducted for the “slicer protein” Argonaute (Ago) [3]. Silencing of genes by RNAi interference is a powerful method to study gene function in both prokaryotic and eukaryotic organisms [2]. Therefore, this method has been widely used to study the biology of multiple parasites and pathogens [4]. Some of these studies have even led to rapid identification of targets needed for drug and vaccine development [5]. However, the main limitation of this method lies in the fact that some organisms do not have the RISC enzymes and, thus, siRNA is not feasible [6–8]. Genomic data demonstrated that *Cryptosporidium* does not have RISC machinery. Therefore, the RISC-dependent silencing method cannot be used to study gene function in this organism [7, 8]. To circumvent this problem, we recently developed a novel alternative method for silencing genes in this parasite [9].

Previous studies demonstrated that human Argonaute (hAgo2) associated with ssRNA induced specific degradation of mRNA targets in vitro [3]. We hypothesized that the recombinant human Ago (hAgo2) could be loaded with *Cryptosporidium* ssRNA to form a hybrid complex, then we pondered that if we could introduce this complex into live parasites then it should bind specifically mRNA target and then produce specific silencing due to the slicer activity of enzyme Ago2 (Fig. 1). Since bioactive proteins can be transported efficiently into cells using protein transfection reagents (PTR), first we demonstrated the feasibility to introduce proteins into the parasites using PTR [9]. After these experiments, we coupled *Cryptosporidium* ssRNA and hAgo2 and showed that transfection of complexes into parasites did not affect parasite viability nor sporozoites excystation. The next step in the development of the method was to show the feasibility of silencing specific genes in *Cryptosporidium* by using preassembled ssRNA–hAgo2 complexes [9]. For these experiments, two sporozoite genes were selected as targets: *Cryptosporidium parvum* surface protein of 15 kDa (Cp15) and calcium-dependent kinase (CDPK1). Both of these molecules play key roles during the invasion process. Also, we targeted a glycoprotein gene gp900, which is not expressed during the invasive stage. We transfected oocysts with ssRNA–hAgo2 complexes targeting Cp15, CDPK1, and gp900, and after 4 h of transfection, we evaluated the effect of silencing by analyzing the expression of mRNA target by reverse transcriptase PCR (RT-PCR). All three ssRNA–hAgo2 complexes reduced expression of the target genes (~70–90%). No reduction was observed when we treated parasites with scrambled ssRNA or PRT alone. In these experiments, we conducted western blot analysis which confirmed that silencing correlates with reduction of the protein [9]. Once we demonstrated gene silencing in parasites, then next question was to show the usefulness of the method to evaluate the role of targeted genes in a biological process. Therefore, we developed an invasion assay model to evaluate the effect of silencing during the infection of human epithelial cells (HCT-8) cultured in the laboratory. The hypothesis was that silencing of essential genes would block entrance of the parasite to the cell which may lead into a reduction in the number of parasites in HCT-8 evaluated by RT-PCR. Previous reports showed that inhibition (with antibodies and drugs) of *Cryptosporidium* Cp15 and CDPK1 led to a reduction of parasite invasion in host cells [10, 11], we hypothesized that silencing of these genes would reduce parasite invasion. By contrast, no reduction was expected with silencing of gp900, since this protein is involved in oocyst formation. As predicted, silencing of Cp15 and CDPK1 led to a 70% and 60% reduction, respectively, in the number of parasites found inside host cells compared with infection with nontransfected parasites. By contrast, silencing of gp900 did not significantly reduce parasite numbers. Overall, these data demonstrate that our siRNA methods can be used to evaluate the phenotype of targeted genes during infection [9].

The major gap addressed by our novel methodology is the lack of methods to identify novel drug targets, which are desperately needed for the treatment of cryptosporidiosis. The goal of this chapter is to describe in detail the methodology to silence genes in *Cryptosporidium* [9]. The novel aspects of our method include the use of preassembled hybrid complexes of human Ago2 and *Cryptosporidium* ssRNA to suppress gene expression and the transfection of *Cryptosporidium*. The major advantage of our method over existing technology (i.e., CRISPR-Cas systems) is the simplicity and speed of analysis of the silencing. The use of

preloaded Ago–ssRNA complexes “ready-to-silence” completely bypasses the transcription–translation process associated with gene expression, reducing the time until the cells can be assayed from days to less than 2 h. This may allow scale-up of the method for high-throughput analysis. Therefore, we anticipate the method described here will be useful to identify novel targets and novel functions for genes with previously unknown functions in *Cryptosporidium*.

2 Materials

1. Recombinant protein human Argonaute 2 (hAgo2) from human (Sino Biologicals, North Wales, PA).
2. RNase-free water.
3. Assembling buffer: 30 mM HEPES, pH 7.4, 150 mM KOAc, 2 mM Mg(OAc)₂, 5 mM dithiothreitol (DTT).
4. Protein transfection reagent (PTR): Pierce™ Protein Transfection Reagent (Thermo Fisher Scientific, Inc., Waltham, MA).
5. HEPES-buffered saline (HBS) for PTR: 10 mM HEPES, 150 mM NaCl, pH 7.0.
6. Acidic water: H₂O at pH 2.5–3.
7. Excystation media: RPMI 1640 medium, 1× antibiotic/anti-mycotic mix, and 0.8% taurocholic acid sodium salt hydrate. The media is filtered through a 22-μm filter.
8. qRT-PCR kit: qScript™ One-Step SYBR® Green qRT-PCR Kit, Low ROXTM (Quanta BioSciences/VWR, Radnor, PA).
9. PCR amplification plate: MicroAmp® Fast 96-well (Applied Biosystems, Foster City, CA).
10. PCR machine: qPCR in a 7500 Fast Real-Time PCR System (Applied Biosystems).
11. PCR optical adhesive film for 96-well PCR plates.
12. RNA purification kit: RNeasy plus mini kit (Qiagen, Germantown, MD).

3 Methods

3.1 Preparation and Design of Single-Stranded RNA (ssRNA)

1. We design the ssRNA using computational software and synthesize the ssRNA from a commercial vendor (*see Note 1*).
2. Resuspend ssRNA at 100 mM with RNase-free water (*see Note 2*).
3. Dilute the sample 1:10 with RNase-free water and store at –20 °C until use.

3.2 Complex Assembling (ssRNA–hAgo2)

1. Linearize the ssRNA: Incubate the tube (with the diluted ssRNA) for 1 min at 95 °C.
2. Assembling ssRNA–hAgo2 complexes (Table 1): Mix in a microcentrifuge tube 250 ng (2.5 µL) of recombinant hAgo2 (*see* Note 3) and 2.5 µL of the respective ssRNA previously diluted (*see* Subheading 3.1), add 15 µL of the assembling buffer and mix well with pipette (triturate).
3. Incubate the sample at room temperature for 1 h.

3.3 ssRNA–hAgo2 Complex Encapsulation With Protein Transfection Reagent (PTR)

1. Add 15 µL of PTR into the 1.5 mL tube that contains the assembling reaction (prepared as above in Subheading 3.2) and mix the encapsulation reaction (Table 2) by triturating.
2. Incubate the sample for 30 min at room temperature.

3.4 Parasite Transfection

1. Transfer 5×10^5 *Cryptosporidium* oocysts (*see* Note 4) to a 1.5-mL tube, add equal volumes of water (RNase free), and triturate.
2. Centrifuge the sample at $500 \times g$ for 10 min. Discard the supernatant in a plastic container containing ~250 mL of water containing 6% bleach.
3. Resuspend the pellet in the required amount of RNase-free water. For each tested sample, use the ratio of 5×10^5 oocysts resuspended in 5 µL, then multiply this volume for the total number of samples (*see* Note 4).
4. Prepare transfection reaction (Table 3) as follows: Transfer 5×10^5 oocysts (5 µL) to a reaction tube (1.5 tube) that contains the assembled complex and the protein transfection reagent described above. Note: After transferring the parasites, mix the sample by triturating to resuspend parasites settled at the bottom of the tube.
5. To allow transfection, incubate the sample for 1 h at room temperature.
6. To activate the silencing, incubate the sample for 2 h at 37 °C.
7. In preparation for RNA extraction or excystation assays, centrifuge parasites for 1 min at maximum speed, and discard the supernatant.
8. Silencing analysis is performed by extracting the total RNA. Lyse the parasites for RNA extraction by adding 350 µL of RLT buffer contained in the RNeasy Plus Mini Kit as described in Subheading 3.6 (*see* Note 5).

3.5 Parasite Excystation

1. Resuspend the pellet of treated parasites in 25 µL of acidic water (pH 2.5) and incubate on ice for 10 min.
2. Add 250 µL of excystation media to the tube with the parasites.

3. Activate the excystation by incubating the samples for 1 h at 37 °C.

3.6 RNA Extraction

1. Resuspend transfected oocysts in 350 µL of RLT buffer included in the RNA isolation kit (*see* Note 6).
2. Before starting RNA extraction, heat the sample for 2 min at 95 °C.
3. Remove genomic DNA by transferring the heated 350 µL to the genomic DNA (gDNA) separator columns provided in the RNA extraction kit.
4. Centrifuge the samples for 30 s at $>8000 \times g$ and recover the elution in a collection tube. Discard the genomic DNA column and save the flow-through in collection tube for further processing.
5. Add 350 µL ethanol (70%) to the flow-through in the collection tube and mix well (triturate).
6. After mixing, transfer a maximum of 700 µL to the RNeasy column, and centrifuge the sample at $>8000 \times g$ for 30 s. Discard the elution from collection tube (provided in the kit).
7. Place column in elution tube.
8. Wash the column with 700 µL of RWI buffer and centrifuge as before. Discard the elution.
9. Wash the column with 500 µL of RPE buffer and centrifuge as before. Discard the elution.
10. Wash the column a final time with 500 µL RPE buffer, but then centrifuge at $>8000 \times g$ for 2 min. Discard the elution.
11. Transfer the empty RNeasy column to a new collection tube and centrifuge for 1 min at maximum speed to remove ethanol traces.
12. Transfer the RNeasy column to a new 1.5-mL microcentrifuge tube, and add 100 µL of RNase-free water to detach and elute the RNA from the silica membrane.
13. Measure the RNA concentration of the sample by spectrophotometry or using a Nanodrop.
14. Dilute RNA samples at 5 ng/µL (*see* Note 7).

3.7 Primer Design for mRNA Target Amplification by RT-PCR

1. For designing primers, we use computational software (*see* Note 8). Target and primers sequences of ssRNAs used as controls for silencing are indicated in Table 6.

3.8 Evaluation of Silencing by RT-PCR (See Table 4)

1. Add a total of 2 µL of purified RNA template to a 96-well reaction plate (0.1 mL).

2. Prepare RT-PCR mix as follow: 5 μ L of the One-Step SYBR Green Master Mix, Low Rox (2 $\times$), 0.25 μ L of each primer at a 10 μ M concentration, 0.25 μ L of the reverse transcriptase, and 4.25 μ L of nuclease-free water for a total of 10 μ L of mix per sample.
3. After RNA is loaded, add 10 μ L of the reaction mix to each well containing 2 μ L of RNA sample, for a total of 12 μ L per well.
4. Seal the plate with PCR optical adhesive film.
5. Run the reactions on a real-time PCR system with the following cycling conditions:
 - 50 $^{\circ}$ C for 15 min.
 - 95 $^{\circ}$ C for 5 min.
 - 95 $^{\circ}$ C for 15 s, 63 $^{\circ}$ C for 1 min for 50 cycles.
 - Melting point analysis (95 $^{\circ}$ C for 15 s, 60 $^{\circ}$ C for 1 min, 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 15 s).
6. Normalize the results for each target with a housekeeping gene (e.g., GAPDH, see Table 6) to probe for parasite counts.
7. For analysis, use the ddCt calculation method to compute the relative gene expression in all samples. Results are shown by the average for each target with the respective standard deviation. The calculation steps are as follows:
 - Determine the averages and standard deviation from the PCR replicates for all samples.
 - Normalize all targets to the housekeeping gene Ct value of the control:

$$dCt_{GAPDH} = Ct_{GAPDH}^{Control} - Ct_{GAPDH}^{Gene}$$
 - Normalize the Gene Ct values by adding the dCt_{GAPDH} value to the corresponding Gene Ct_{Target} values. This will be the dCt_{Target} :

$$dCt_{Target} = Ct_{Target}^{Gene} + dCt_{GAPDH}^{Gene}$$
 - Calculate the ddCt by subtracting the $dCt_{Target}^{Control}$ from the dCt_{Target}^{Gene} :

$$ddCt_{Target}^{Gene} = dCt_{Target}^{Gene} - dCt_{Target}^{Control}$$
 - Calculate the fold change:

$$FC = 2^{\Delta - ddCt_{Target}}$$

4 Notes

1. We used computational software to design ssRNA of 17–23 nt complementary to *Cryptosporidium* mRNA targets. We synthesize the ssRNA with the following modifications: 5' prime is phosphorylated and 3' prime is tailed with dTdT. Negative controls may include (17–22 nt) unrelated ssRNA sequences, sense RNA, or scrambled RNA. Positive controls are listed in Table 5.
2. To prepare ssRNA stock at 100 mM, centrifuge the tube with the lyophilized ssRNA for 5 min at maximum speed. To calculate the amount of water to resuspend ssRNA at 100 mM, first we identify the number of nmol indicated in the tube, then we use the following formula: number of mM = μL of RNase-free water to be added.
3. For assembling protocol, resuspend the lyophilized hAgo2 at 125 mg/ μL with 160 μL ultrapure RNase-free water. Aliquot the protein and store at $-80\text{ }^{\circ}\text{C}$ until use.
4. For silencing experiments, we recommend using fresh *C. parvum* oocysts (up to 1 month old). On the day of the experiments, the viability of the oocyst is tested by the excystation assay as follows. We only use oocysts with >80% excystation rate determined by microscopy. For the transfection experiments, the parasites can be prepared during the encapsulation time (30 min) described in the encapsulation section. Initially, *Cryptosporidium* oocysts (1×10^8) are diluted in 1 mL of PBS/Tween 20 solution (stock solution). We calculate the total number of parasites for our experiment as follows: For each tested sample, we use 5×10^5 oocysts, then we multiply this number by the total number of treated samples (including duplicates, untreated samples, and negative controls). For example, the total number of oocysts needed for 20 samples would be calculated as follows: $(5 \times 10^5 \text{ oocysts}) \times 20 = 100 \times 10^5 = 1 \times 10^7$ total oocysts.
5. For silencing experiments, the effect can be evaluated before the excystation and/or after excystation. If the RNA is isolated after the excystation, then skip to the excystation section.
6. RNA extraction is conducted with the Qiagen's RNeasy Plus Mini Kit following the instructions listed in the quick protocol included in the kit.
7. After extraction, the sample can be stored at $-20\text{ }^{\circ}\text{C}$ until qPCR analysis.
8. For designing, we analyze full *Cryptosporidium* mRNA sequences obtained from GenBank and CryptoDB. For primer positions, we select regions that flank a 17- to 22-nt splicing region. Primers for qPCR of positive controls are indicated in Table 6.

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Fig. 1.

Silencing genes in *Cryptosporidium*. (a) Assembling: hAgo2 protein is loaded with *Cryptosporidium* ssRNA complementary to mRNA target. (b) Encapsulation: hAgo2–ssRNA complex is encapsulated within liposomes (protein transfection reagent). (c) Transfection: *Cryptosporidium* oocysts are transfected with complexes. (d) Silencing: hAgo2–ssRNA binds to mRNA target, translation is blocked, and mRNA target is sliced by hAgo2, then expression of target is reduced

Table 1

Assembling reaction

Components	Positive control (μL)	Negative control (μL)	Sample (μL)
ssRNA (1:10)	2.5 (<i>see</i> Table 5)	2.5 (<i>see</i> Table 5)	2.5
Human Argonaute protein 2	2.5 (250 ng)	2.5 (250 ng)	2.5 (250 ng)
Assembling buffer	15	15	15
Total volume	20	20	20

Table 2

Encapsulation reaction

Components	Positive control (μL)	Negative control (μL)	Sample (μL)
Assembling reaction components	20	20	20
Protein transfection reagent	15	15	15
Adjust to 35 μL with RNase-free water	0	0	0
Total volume	35	35	35

Table 3

Transfection reaction

Components	Positive control (μL)	Negative control (μL)	Sample (μL)
Parasites	5×10^5 (5 μL)	5×10^5 (5 μL)	5×10^5 (5 μL)
Acidic water, pH 2.5	25	25	25
Excystation media	250	250	250
Total volume	280	280	280

Table 4

RT-PCR silencing reaction

Components	Positive control (μL)	No RNA control (μL)	Sample (μL)
RNA sample	2 (Table 5)	2 (Table 5)	2
One-Step SYBR Green Mix, Low ROX (2×)	5	5	5
qScript One-Step reverse transcriptase	0.25	0.25	0.25
Forward primer	0.25	0.25	0.25
Reverse primer	0.25	0.25	0.25
Nuclease-free water	4.25	4.25	4.25
Total volume	12	12	12

Table 5

ssRNA sequences

Single-Strand antisense RNA	Sequence
^a ssCDPK1 ^{a,g}	5' rCrArCrUrUrCrCrUrUrCrUrUrCrUrUrCrUrCrCrC TT 3'
^{b,g} ssActin	5' rCrArUrArUrGrArArCrUrArCrCrArArUrGrGrC 3'
^{c,g} ssAp2	5' rGrCrUrArGrUrGrGrArUrArUrArGrGrGrArUrGrC 3'
^{d,g} ssNDK	5' rArArArCrUrArGrCrCrCrArCrArArUrCrCrArCrG 3'
^e ssRom1	5' rUrArArCrGrArCrUrArCrUrUrGrArArUrGrGrG TT 3'
Scrambled (negative control)	^f 5' rGrGrGrUrCrArCrGrUrGrCrGrArArCrArUrGrA 3'

^a ssCDPK1: ssRNA for calcium-dependent kinase 1^b ssActin: ssRNA for actin^c ssAp2: ssRNA for Apicomplexan transcription factor 2^d ssNDK: ssRNA for nucleoside diphosphate kinase^e ssROM1: ssRNA for Rhomboid protein 1^f ssRNA scr: scrambled RNA used as negative control^g ssRNA sequences that induce silencing >70% by RT-PCR

Table 6

Target genes and primer sequences used for RT-PCR analysis

Target	Accession number	Primer sequence
GAPDH ^a	XM_627718	Forward: 5'-TGA CCC ATC AAA ATT CAA CG-3' Reverse: 5'-ACG GAA ACA TCT GGA GTT GG-3'
CP23 ^a	XM_625938	Forward: 5'-CAA TCA GCA ACC AAG CTC AA-3' Reverse: 5'-TTG TTG AGC AGC AGG TTC AG-3'
CDPK1 ^b	XM_001388059	Forward: 5'-GCA GTA GGG AAT ACA GGA ACA A-3' Reverse: 5'-CCG CTA CCA CTC TGA AGA AA-3'
Actin ^b	FX115681	Forward: 5'-CAT TGC TTT AGA TTA CGA GGA AGA-3' Reverse: 5'-TTG GAA TAA AGC CTC AGG ACA-3'
Ap2 ^b	XM_627219	Forward: 5'-TAA ACA AGC CTG AGC CAA CC-3' Reverse: 5'-TCG GTT CCA ATT TCA TGT CC-3'
NDK ^b	FX115659	Forward: 5'-GCA ACT CGA CTG AAT CGT CA-3' Reverse: 5'-CTT TCC TGC ATG ATC GGA AT-3'
GP900 ^c	AF068065	Forward: 5'-GCA CCA TTT GAG TTG GAT GTT AG-3' Reverse: 5'-AGT TGG GAG ACC AGT GAT AGA-3'

^aPrimers for housekeeping genes (nonsilenced) used for normalization^bPrimers for positive ssRNA controls used for silencing^cPrimers for nontargeted gene during silencing (i.e., silencing with ssNDK does not affect GP900 expression in treated parasites vs. WT)